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SIMULTANEOUS DETECTION OF MULTIPLE DISEASE STATES

FINAL REPORT

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13. ABSTRACT *(Maximum 200 words)*

The objectives for thisproject were to demonstrate the utility of BioStar Optical ImmunoAssay (OIA)^{tm} for the detection of infectious agents in insect populations using Malaria and Lyme antigens as models. Also to demonstrate OIA applicability for the diagnosis of host response to malarial infections.

To meet the latter objective a recombinant antigen specific to P.falciparum was immobilized to a propertiary solid support, which provided the desired optical properties, and samples assayed for the presence of antibody to this antigen. Specificity of this antibody response was clearly demonstrated.

For the detection of Malaria or Lyme antigens, the appropriate monoclonal antibodies were immobilized to the optical solid support. Samples were examined for the presence of antigen in heat-inactivated organisms or infected carriers (mosquitos or ticks). Developed extraction protocols allowed unifected organisms to be assayed without the generation of a signal.

OIA's flexibility allows any number of analytes to be assayed in a panel forma And due to its simplicity, OIA has been demonstrated to be generally applicable to a wide range of testing environments. Since unstable reagents are not generally employeed there are no special storage requirements for field tests.

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Purpose and Scope of Project:

The objective of this project was to establish the efficacy of the Interference Slide Optical ImmunoAssay (OIA)™ in the simultaneous detection of three separate reagents. Specimens will be tested for *Plasmodium falciparum* circumsporozoite (CS) antigenic determinants in mosquitoes, an antibody response to the CS antigen in human serum samples, and *Borrelia burgdorferi* in ticks. Several different ligand coating techniques and nonspecific blocking schemes were evaluated. Extraction protocols for *Anopheles* mosquitoes and deer ticks (*Ixodes dammini*) were examined. Finally, assay performance attributes: sample matrix (buffers, volume, concentration), operating temperature, and incubation times were optimized. The assay formats were initially optimized on ellipsometric wafers and then transferred to interference wafers.

PROJECT OBJECTIVES:

Originally, this study was proposed to demonstrate the simultaneous detection of three different autoimmune markers (rheumatoid factor, anti-cardiolipin, and anti-dsDNA). In accordance with Dr. Wirtz's request, the focus of the project was shifted to developing assays for the detection of infectious agents or host response to infectious agents. The specific aims of this Phase I project were:

1. Demonstrate the capacity to immobilize the three different ligands individually, as three discrete zones for simultaneous sample testing.

2. Demonstrate distinct, specific positive visual responses for each analyte.

3. Demonstrate assay sensitivity (individually).

INTRODUCTION TO OIA AND ELLISPOMETRY/GRAY SCALE TECHNOLOGIES

OIA is a direct physical detection methods that exploit the optical properties of thin films to detect biochemical reactions without the need for a molecular tag such as radioactivity or fluorescence. As in all immunoassays, the method of signal generation is a distinguishing feature of the assay. OIA may be divided into two distinct formats on the basis of signal generation: visual and instrumented. In both cases monocrystalline silicon wafers are employed as the substrate on which the assay is performed. Visual OIA exploits classical thin film optics to generate a perceived color change as the direct result of the immunological binding reaction. Instrumented OIA assays utilize the Sagax Comparison Ellipsometer to accurately measure a change in film thickness at the surface of the silicon wafer.

1. Visual OIA

The precise method of signal generation for visual OIA exploits the behavior of light reflected at the boundaries of thin films and destructive interference. When light impinges a boundary of a thin film, as defined by a change in refractive index, it undergoes reflection and refraction according to Snell's Law, ie. a portion of a given wavelength of the incident light is reflected at that boundary and the remainder penetrates the film to be reflected/refracted at subsequent boundaries. This partitioning of light is a function of the wavelength of incident light and the refractive index of the film. In a simple system with one film on a reflective substrate, a portion of the incident light is reflected at the air-film interface and a portion at the film-substrate interface.

The second reflection is essentially phase-delayed with respect to the initial reflection. The amount of delay is a function of the film thickness and its refractive index. When the delay of a given wavelength is a whole number multiple of 180° and the intensity of the two reflections are approximately equal, that wavelength of the incident spectrum undergoes destructive interference and is essentially removed from the reflected light. This type of film, known as an anti-reflection (AR) film, is employed in the visual OIA where the suppressed wavelength is in the visible spectrum. Due to the nature of the anti-reflection film, when the thickness of the film changes the visually perceived color changes by shifting the wavelength of suppressed reflected light. When a visual OIA testpiece, comprised of a reflective substrate, an appropriate AR film to which is attached a biological ligand, is viewed in ambient light the surface appears colored as a result of the destructive interference. The testpiece may then be exposed to a sample suspected of containing a specific analyte. If the analyte is present, binding to the biological ligand at the surface will occur this changes the effective AR film thickness which results in a different visual color. This color change provides a direct physical detection of the binding reaction.

In visual OIA the AR film consists of a dielectric material, usually a metal oxide or nitride, which has been deposited on the silicon wafer by techniques developed for the semiconductor industry. These films are readily produced in large quantities to extremely high tolerances at low cost by this preexisting technology. Immobilization of biological ligands, antibodies, or antigens to these surfaces may be accomplished by either passive or covalent techniques. In general, covalent techniques are preferred for antibodies and antigens by virtue of their stability and reproducibility. Due to the limited surface area available on the monocrystalline silicon wafers, attention must be paid to efficient immobilization to ensure sufficiently reactive surfaces. BioStar has invested a great deal of time investigating traditional and innovative immobilization techniques for these surfaces. Traditional chemistries, such as silane attachment, have proven to be of limited utility, while newer solutions with polymer mixtures have demonstrated broader applicability.

2. Instrumented OIA

Instrumented measurement of the OIA testpiece employs the use of the Sagax Comparison Ellipsometer. This instrument operates on the basis of comparing the degree of elliptical polarization, caused by the reflection of plane polarized polychromatic light, between two surfaces. Incident polychromatic light is columnated and plane polarized. The polarized light is reflected at an oblique angle from the reference surface, a reflective substrate with the same optical characteristics as the testpiece. The reflected light is then elliptically polarized as a result of reflection. The elliptically polarized light then reflects from the test surface. The test surface and reference surface are arranged perpendicular to one another such that after reflection from the test surface, the light is once again plane polarized, if the reference and testpieces are of identical thickness and refractive index. If their thickness and/or refractive indices are not identical, the light retains some elliptical character, the greater the difference the greater the elliptical character. A second polarizer is then used to filter the light which removes the plane polarized light corresponding to identical films. An increase in elliptical character will result in greater light transmition through the second polarizer. Thus, a change in thickness has been transformed into a change in light intensity which may then be measured using conventional techniques. Employing the Comparison Ellipsometer in this fashion resolution to +/- 1 angstrom may be achieved. The measurement scale employed with the Comparison Ellipsometer is called Gray Scale which uses an immage processing program with an 8 bit processor. Total light intensity variation is divided into 256 units called gray scales which are analogous to absorption values and OD measurements.

Unlike conventional ellipsometry, the Comparison Ellipsometer is designed to allow broad field measurements. This feature allows simultaneous measurement of the entire reaction zone to be performed, so measurement errors do not arise because of non-homogeneous binding or reaction patterns. Because the surfaces of the interference slide and the ellipsometric slide are identical chemically, an assay can be developed in one format and transferred to the other essentially unchanged.

Tasks and Results:

Task 1 was devoted to assessing possible problem areas, through a literature review, of the newly defined scope of research. The remainder of this section presents a summary of the results obtained for tasks completed in Performance Periods number 2 to 5. This summary is an abbreviation of the results presented in the monthly technical reports. Results from the final Performance Period (#6) are discussed in greater detail. The discussion is separated into a complete review of the data collected for the lyme antigen (Ag) detection assay, Periods 2-6, followed by a review of the data obtained for the malaria Ag detection assay, Periods 2-6, and finally a review of the malaria antibody (Ab) detection data, Periods 2-6.

Note: All coating protocols discussed are considered confidential.

LYME ANTIGEN DETECTION

The negative controls used in this system were deer ticks chosen from a group of "uninfected" ticks, as demonstrated by dark-field microscopic examination. The positive controls were heat-killed, lyophilized *B. burgdorferi* spirochete samples (10 mg total protein/ml); the actual antigen concentration was not available. The lyme antigen was reconstituted in a 1:1 (v/v) mixture of deionized water (DI) and glycerol. It was then aliquoted into small containers and stored at 0°C. Once thawed they were stored at 4-8°C. Uninfected ticks were used as negative controls in the lyme Ag detection assays and were prepared by triturating in 50 ul Blocking Buffer (BB):NP-40.2.

PERIOD 2:

Assay Conditions:

The assay conditions selected were optimized in prior investigations of several other systems. Five microliters of the controls (positive or negative) were pipetted onto the ligand-coupled wafer surfaces and incubated for three minutes on a 45° C heating block. The spots were rinsed with water, dried in a stream of nitrogen and evaluated visually and/or using the comparison ellipsometer. This protocol was followed for all of the assays discussed below.

Antibody Coating/Immobilization Protocols:

BioStar examined a variety of antibody coating protocols using polybutadiene coated wafers (see Progress Report #2). These methods included, antibody coating concentration was a constant 20 ug/ml:

1) Pre-treating the wafers for two hours at room temperature with Omnibind A/G , InFerGene Co., Benicia, CA, (25 ug/ml, 0.1% glutaraldehyde, PBS). Omnibind A/G, a protein A/G recombinant hybrid which binds the Fc portion of IgG, should facilitate the coating and orientation of the monoclonal antibodies (MAbs). The MAbs in 1:1 (v:v) deionized water: glycerol (20 ug/ml final coating concentration) were then added directly to the solution and incubated for two hours at room temperature.

2) Incubation with MAbs in PBS with 0.1% glutaraldehyde for two hours at room temperature.

3) Incubation with MAbs in PBS with 0.1% glutaraldehyde for 24 hours at room temperature.

4) Incubation with MAbs in PBS with 0.1% glutaraldehyde and 1% BSA (bovine serum albumin) for 2 hours at room temperature.

5) An immobilization approach which employed smaller volumes of reagents was also examined. Polybutadiene wafers were coated by incubating the MAbs solution between two wafers with the optical surfaces in direct contact. The wafers were incubated in a humidified, 37°C incubator for one hour. The ligands were crosslinked by incubating the wafers for 10 minutes in a 37°C, saturated glutaraldehyde, vapor incubator.

Given the hydrophobic properties of the polybutadiene (PBD) surface, the ligand-coupled wafers were treated with blocking reagent(s) to reduce non-specific binding of non-analyte materials to unreacted organic functional groups much the same as in RIA, EIA and ELISA formats. Findings to date indicate BioStar has successfully blocked unreacted activation sites by incubating the ligand coated wafers in a PBS/1% BSA/2% sucrose/1% glycerol solution for 2 hours at room temperature.

Results:

Samples of heat inactivated *B. burgdorferi* reconstituted in DI/glycerol yielded undetectable signals both visually and upon examination with the comparison ellipsometer. It was suspected that the glycerol in the reconstitution solutions was adversely affecting the Ab/Ag reaction. New lyophilized samples were reconstituted in DI water as follows: heat-inactivated *B. burgdorferi* (10 mg total protein/ml). All slides were examined with the comparsion ellipsometer and the results are reported in terms of gray scale units.

DETECTION OF HEAT INACTIVATED B. burgdorferi ANTIGEN/LYME ANTIGEN

COATING PROTOCOL	MAb COATING TIME	MAb COATING THICKNESS[a]	GRAY SCALE[b] FOR LYME ANTIGEN POSITIVE BINDING
#1	2 hours	30	25.32
#2	2 hours	29	43.67
#3	24 hours	50	20.08
#4	2 hours	3	76.16
#5	1 hour	64	44.50

[a] Values given are in angstroms and were obtained with a Gaertner absolute ellipsometer.
[b] Sample applied was a 5 microliter sample of the stock *B. burgdorferi* spirochetes containing the reactive surface antigen.

Note: The extracted negative tick samples gave consistantly low gray scale values (6.63 or less).

Based on these results it was clear that this assay was not improved by the use of OmniBind A/G wafer pre-treatment. Therefore, subsequent experimentation did not employ Omnibind A/G pre-treated wafers. While addition of BSA to the coating solution for the anti-*B. burgdorferi* increased the coating efficiency, it did not seem to do so for the antibody to *P. falciparum* (see below). Of the coating protocols examined to date, the solution based methods would appear to produce the most reactive surface. Continued examination of these methods and of alternate surface materials will yield more reactive antibody coats. Given the successful results generated on the ellipsometric PBD wafers, the coating techniques and assay protocols were transferred to interference PBD wafers for the lyme Ag detection assays. The MAb anti-*B. burgdorferi* was coated between two wafers using the fifth immobilization technique described above. The blocking, sample, temperature and incubation conditions were all held constant. In the lyme test we decided to try pipetting 5 ul of MAb anti-*B. burgdorferi* (a monoclonal-monoclonal sandwich) on top of an assayed *B. burgdorferi* spot to see if it would enhance the signal generation. The gray scale results are shown below:

SAMPLE	GRAY SCALE
I. dammini in BB:NP-40	0.00
heat-inactivated *B.burgdorferi* (in DI)	21.79
B. burgdorferi + MAb anti-*B. burgdorferi*	57.62

PERIOD 3:

<u>Coating/Immobilization:</u>

Polybutadiene polymer surfaces were generated as described above. Additionally, Sera-Coat beads, (Seradyn, Indianapolis, IN) were evaluated due to their successful application in a separate OIA diagnostic format BioStar is developing. Sera-Coat beads are an acid-modified, styrene-based copolymer containing carboxylic acid groups for covalent coupling of ligands.

Two different formulations of Sera-Coat beads were evaluated: TC 3X and TC 7X; the main difference between the two being the ratio of the styrene copolymer. The carboxylic acid content is the same for both preparations. Beads were diluted 1:50 in methanol and spin coated onto silicon wafers. After spin coating wafers were incubated at 37° C for 2 hours to ensure adhesion to the silicon surface.

The carboxyl groups were activated by incubating the wafer for one hour at room temperature in 0.5% N-hydroxysuccinimide: 0.5% water-soluble carbodiimide (final concentration) in 0.1 M sodium phosphate, pH 5.6 after which the wafer is rinsed under deionized water. Monoclonal anti-*B. burgdorferi* [80 ug/ml in PBS] were incubated in solution between two wafers with the optical surfaces in direct contact. The wafers were incubated in a humidified, 37° C incubator for one hour. Subsequent covalent cross-linking of the Ab/Ag Sera-Coat wafers by incubating them for 30 minutes in a 37° C, saturated glutaraldehyde (GA), vapor incubator was examined for its effect on the thickness of the ligand layer and/or signal strength.

Four different blocking reagent(s) were evaluated for their effect in reducing non-specific binding of non-analyte sample materials to unreacted organic functional groups on the Sera-Coat wafers. The blocking reagents analyzed were: PBS/1% human serum albumin, PBS/5% normal human sera, PBS/5% chicken sera/0.1% Tween 20/0.1 mM thimerosal, and PBS/1% BSA/2% sucrose/1% glycerol. All of the blocking reagents were incubated with the Ab/Ag coated wafers for 2 hours at room temperature.

Sample Preparation:

The *B. burgdorferi* samples, have been heat-inactivated. How this treatment process affects the quantity and quality of antibody-reactive antigen is not known. Uninfected ticks were extracted in a buffer containing chitinase, paralleling investigations examining the addition of chitinase to the mosquito extraction buffers in an effort to reduce nonspecific background (see period 3, malaria Ag detection, p. 10). These preparations were then compared with samples extracted in BBNP-40 alone. Whole ticks, were individually ground up in 0.05 ml of BBNP-40. The pestle was rinsed with 0.1 ml of BBNP-40 then 0.15 ml chitinase (3.33 units/ml in 0.1 M sodium citrate, pH 5.5) was added. This mixture was incubated overnight at room temperature with gentle agitation.

Assay Conditions:

As mentioned in the period #2 section, preliminary investigations of other systems had optimized the assay conditions for five micro-liter samples incubated for three minutes on a 45° C heating block. The lyme Ag detection assays are yielding the best signals under these conditions. Five microliters of heat-inactivated *B. burgdorferi* was assayed at 45° C for 3 minutes, then 5 ul of MAb anti-*B. burgdorferi* (same as capture MAb) was assayed identically on top of the same spot. Or the sample and MAb were incubated together for 5 minutes at RT prior to the 45° C, 3 minute assay.

Results:

In period #2 we discussed the MAb-MAb sandwich assay in which 5 ul MAb anti-*B. burgdorferi* was pipetted on top of an assayed *B. burgdorferi* spot. The data then suggested signal enhancement. Signal amplification might be explained by the possibility of numerous antigenic determinants on the cell surface of *B. burgdorferi* to which the secondary MAb bind. However, experimentation demonstrated that MAb anti-*B. burgdorferi* assayed alone on the MAb anti-*B.*

burgdorferi coated polybutadiene wafer yields a positive signal and may be nonspecifically enhancing the positive control sample - heat-inactivated *B. burgdorferi*. All slides were examined with the comparison ellipsometer and the results are reported in terms of gray scale units.

SAMPLE	GRAY SCALE
heat-inactivated *B. burgdorferi* (in DI)	30.79
B. burgdorferi + MAb anti-*B. burgdorferi**	27.90
*(simultaneous Ab addition, incubation, then assayed)	
B. burgdorferi + MAb anti-*B. burgdorferi* (sequential)	37.30
MAb anti-*B. burgdorferi* (assayed alone)	31.87

Further evaluation of the monoclonal-monoclonal sandwich system is discussed below. Normal mouse sera and mouse IgG was added to the blocking reagent in an effort to eliminate the nonspecific binding of the MAb anti-*B. burgdorferi* (see period #4).

Uninfected ticks were extracted in a buffer containing chitinase, using the protocols described below for the malaria antigen. This sample was compared to uninfected ticks extracted in BBNP-40 alone and the heat inactivated *B. burgdorferi* sample (see below).

DETECTION OF HEAT INACTIVATED *B. burgdorferi* ANTIGEN/LYME ANTIGEN AND EXTRACTED UNINFECTED TICK SAMPLES

	GRAY SCALE VALUES[a]		
WAFER COAT[b]	LYME ANTIGEN[c]	TICK BB:NP-40	TICK CHITINASE
PBD Interference	59.23	0.0	0.0
TC 3X Ellipsometric	22.49	10.30	2.67
TC 3X Interference	35.26	14.69	9.31

[a] Five microliter samples assayed 3 minutes at 45° C.
[b] Anti-*B. burgdorferi* MAb H5332 [100 ug/ml].
[c] Sample of the stock *B. burgdorferi* spirochetes containing the reactive surface antigen.

The chitinase extracted tick samples yielded lower gray scale values compared with the BBNP-40 extracted samples. In this case, however, the chitinase samples did not degrade the ligand layer as observed with uninfected mosquitoes extracted in a buffer containing chitinase (see malaria Ag detection, period 3, p. 9). Further discussion of the chitinase extraction protocols is discussed below.

PERIOD 4:

Coating/Immobilization:

Polybutadiene polymer surfaces and wafers coated with Sera-Coat beads were generated as described above in period 3. Monoclonal antibody anti-*B. burgdorferi* [100 ug/ml in PBS] were coated on the wafers as described in the period 2.

Assay Conditions:

All the results reported were from samples assayed at 45° C for three minutes.

The relationship between extraction buffer components and non-specific background binding in uninfected ticks has also been found to display similar attributes as those discussed above. Previously we have discussed the MAb-MAb sandwich assay for lyme Ag detection assay. The incorporation of capture Ab as a secondary Ab has been utilized in various immunoassay formats as a means to increase sensitivity. This technique may improve sample visualization in the OIA format. As reported in the period 3, MAb anti-*B. burgdorferi* assayed alone on the MAb anti-*B. burgdorferi* coated polybutadiene wafer yielded a positive signal. The following blocking solutions were prepared and evaluated: PBS/1% BSA/2% sucrose/1% glycerol/10% NMS and PBS/1% BSA/2% sucrose/1% glycerol/5% mIgG. A polybutadiene interference wafer coated with MAb anti-*B. burgdorferi* [100 ug/ml] was cut into two pieces which were then treated with the two blocking agent solutions. Monoclonal antibody anti-*B. burgdorferi* assayed on the two PBD wafer pieces coated with MAb anti-*B. burgdorferi* treated with either NMS or mIgG yielded positive signals. Experiments diluting the secondary Ab, prior to addition to the sample, will examine whether an optimal secondary Ab concentration exists which amplifies the signal with minimal nonspecific background binding.

PERIOD 5:

Coating And Immobilization:

Protocols were as described in previous periods for coating SERA coat wafers or PBD.

Assay Conditions:

Protocols were as described in previous periods.

Ab-Bead Conjugation:

Antibodies were conjugated to the Sera-Coat beads in solution for use as an amplifying reagent in the antigen detection schemes. As the antigens were assumed to be multi-valent and we had only one available antibody, this antibody was used in both the capture layer and the amplifying reagent. A better amplifying reagent could be produced if another antibody was available for this study. Sera-Coat beads (Seradyn, Indianapolis, IN) are acid-modified, styrene-based copolymer beads containing carboxylic acid groups for covalent coupling of ligands. A water-soluble carbodiimide (CDI) was used to activate the carboxyl groups which react with amine groups on the ligand. A volume of MAb anti-*B. burgdorferi* sufficient to yield a final concentration of 20 ug/ml was added to 1 ml of 2.4% TC 7/PBS bead solution. The preparation was activated by the addition of 100 ul of 2% CDI/DI and incubating for 2 hours at RT with gentle agitation.

Monoclonal antibody anti-*B. burgdorferi* conjugated Sera-Coat beads were evaluated as a secondary amplification reagent. Five microliters of *B. burgdorferi* positive control, MAb-conjugated beads and MAb-conjugated beads/positive control were assayed on a PBD-MAb anti-*B. burgdorferi*-coated wafer at RT for 15, 10 and 5 minutes. Under all three conditions the positive control gave no signal and the MAb-conjugated beads gave a signal comparable to MAb-conjugated beads/positive control.

PERIOD 6:

A recent preparation of MAb anti-*B. burgdorferi*-conjugated Seradyne beads was divided into two fractions, one of which was dialyzed in 12-14,000 MW-cutoff tubing, Spectra/Por 2, for 3 hours against PBS, pH 7.0. This would remove the water-soluble carbodiimide used to activate the beads. Both the dialyzed and undialyzed beads were diluted 1:5 in the following reagents: PBS, heat-inactivated *B. burgdorferi*, ATCC strain 35210 and ATCC strain 35211. Five microliter samples of these preparations were assayed for 5 minutes at RT on a PBD interference wafer coated with MAb anti-*B. burgdorferi*. None of the preparations, whether incorporating dialyzed or undialyzed beads, yielded visual signals. The ATCC strains 35210 and 35211 appear to contain a surfactant. Samples which do not contain surfactants normally react with this surface and maintain a high surface tension, these samples wetted the surface in such a manner as to suggest the presence of a surfactant.

Given the positive results observed when assaying samples at 45° C it was hypothesized that these conditions may be denaturing the samples and exposing a previously unavailable antigenic determinant. An aliquot of the heat-inactivated *B. burgdorferi* control was incubated in a 56° C water bath for 3.5 hours. Five microliter samples assayed for 15 minutes at RT on a PBD interference wafer coated with MAb anti-*B. burgdorferi* produced no visual signal. A hyponitrous acid cellular extraction method was performed on the control. Fifty microliters of 2M sodium nitrate and 50 ul of 2M acetic acid were added to 20 ul of the control and incubated for 1 minute at RT. The sample was neutralized to pH 7.0 with 200 ul of 0.66M sodium hydroxide. A five microliter sample assayed for 15 minutes at RT on a PBD interference wafer coated with MAb anti-*B. burgdorferi* and yielded no visual signal.

Finally, the addition of the surfactants NP-40, Tween 20, and Tween 85 to the heat-inactivated *B. burgdorferi* control was examined. The control was diluted 1:2 in PBS/1% NP-40, PBS/1% Tween 20, and PBS/1% Tween 85. When these preparations and the PBS/surfactant diluents(no antigen) were assayed for 15 minutes at RT on a PBD interference wafer coated with MAb anti-*B. burgdorferi* the two samples (positive and negative controls) containing Tween 85 gave visual signals. The remaining diluents gave no signals in any case. Tween 85 gave a visual signal in the absence of antigen persumably due to the adsorption of oleic acid to the PBD surface.

SUMMARY:

For the Lyme antigen detection assay, we have demonstrated the clear differentiation of heat inactivated organisms from the extracted unifected ticks. Uninfected ticks may be processed with the BB:NP-40 extraction method used with the existing ELISA or with the chitinase protocol. Preliminary investigation into methods to amplify the visual response have indicated that the antibody is reactive with the surface and that blocking routines do not substantially modify this interaction. However, once the antibody is attached to a bead for the amplifier, the non-specific interaction with the surface is eliminated. PBD appears to be the preferred surface for this assay.

Areas to be improved in subsequent studies include:

1. The amplification method, bead concentration, alternate antibody/s
2. The extraction protocol; better discrimination between infected and unifected ticks
3. Sensitivity
4. Assay Incubation Conditions
5. Improve Ligand Density

MALARIA ANTIGEN DETECTION

The negative controls used in this system were dried *Anopheles* mosquitoes again chosen from a group of demonstrated "uninfected" mosquitoes. The positive controls were two heat-inactivated and lyophilized strains of *P. falciparum* - strain 7G8 containing 640,000 sporozoites/vial and strain NF54 containing 250,000 sporozoites/vial. Additionally, mosquitoes taken from a collection of "*P. falciparum*-infected" mosquitoes were extracted and evaluated.

PERIOD 2:

RESULTS:

The immobilization protocols investigated for this assay are the same as those described for the lyme antigen detection system. The methods for Period #2 are described above.
The assay conditions are as described in the above Period #2 section.
The uninfected ticks and mosquitoes (head & thorax) to be used as negative controls in the lyme and malaria Ag detect assays, respectively, were prepared for assay by triturating in 50 ul BB:NP-40. 2 Samples of heat inactivated P. falciparum strain NF54 reconstituted in DI/glycerol when assayed yielded results undetectable both visually and upon examination with the comparison ellipsometer. It was suspected that the glycerol in the reconstitution solutions was adversely affecting the Ab/Ag reaction. New lyophilized samples were reconstituted in DI water as follows: *P. falciparum* strain NF54 (250,000 sporozoites/ml), and strain 7G8 (1,280,000 sporozoites/ml). All slides were examined with the comparsion ellipsometer and the results are reported in terms of gray scale units.

DETECTION OF *P. falciparum* STRAINS NF54 AND 7G8 INACTIVATED CS ANTIGEN

COATING PROTOCOL	MAb COATING TIME	MAb COATING THICKNESS[a]	NF54/CS GRAY SCALE[b]	7G8/CS GRAY SCALE[c]
#1	2 hours	35	12.20	20.59
#2	2 hours	32	13.53	52.36
#3	24 hours	45	10.73	28.38
#4	2 hours	11	28.93	48.26
#5	1 hour	63	8.20	43.19

[a] Values given are in angstroms and were obtained with a Gaertner absolute ellipsometer.
[b] Sample applied was a 5 microliter sample of the stock NF54 sporozoites containing CS antigen.
[c] Sample applied was a 5 microliter sample of the stock 7G8 sporozoites containing CS antigen.
Note: Initial extractions of the mosquitoes gave values of 70.09 gray scale units.

Based on these results it was clear that this assay was not improved by the use of OmniBind A/G wafer pre-treatment. Therefore, subsequent experimentation did not employ Omnibind A/G pre-treated wafers. While addition of BSA to the coating solution for the anti-*B. burgdorferi* increased the coating efficiency, it did not seem to do so for the antibody to *P. falciparum*. Of the coating *protoco*'s examined to date, the solution based methods would appear to produce the most reactive surface. Continued examination of these methods and of alternate surface materials will yield more reactive antibody coats.

The *Anopheles* mosquitoes when extracted with the protocol used in the existing ELISA, react very strongly with the OIA surface. In addition, the mosquitoes were extracted in the following solutions:

1) PBS (pH7.4), 1% BSA, 0.5% Casein, 0.01% Thimerosal, 0.02% Phenol red, Blocking Buffer (BB) 2 ;
2) PBS, 1% BSA, 2% sucrose, 1% glycerol, 0.5% NP-40;
3) PBS, 1% BSA, 2% sucrose, 1% glycerol;
4) DI water.

PERIOD 3:

Coating/Immobilization: See the Lyme Antigen Detection Period 3 section above.

Sample Preparation:

Uninfected mosquitoes were extracted in a buffer containing chitinase in an effort to reduce nonspecific background binding[1]. These preparations were then compared with samples extracted in BB:NP-40 alone. Mosquitoes (head/thorax) were individually ground up in 0.05 ml

of BB:NP-40. The pestle was rinsed with 0.1 ml of BB:NP-40 then 0.15 ml chitinase (3.33 units/ml in 0.1 M sodium citrate, pH 5.5) was added. This mixture was incubated overnight at room temperature with gentle agitation.

Assay Conditions:

Preliminary investigations of other systems had optimized the assay conditions for five micro-liter samples incubated for three minutes on a 45° C heating block. The malaria Ag detection assays yielded the best signals under these conditions. Additionally, assays run at 15 minutes in a humidified, 37° C incubator were performed for the assay but were less than optimal.

Reconstituted samples of heat inactivated *P. falciparum* strain 7G8 (1,280,000 sporozoites/ml) when assayed at RT for 5 minutes yielded results undetectable both visually and upon examination with the comparison ellipsometer. Therefore, at this stage of the study both assays are conducted at 45° C for 3 minutes.

DETECTION OF *P. falciparum* STRAIN 7G8 INACTIVATED CS ANTIGEN AND EXTRACTED MOSQUITO SAMPLES

	GRAY SCALE VALUES[a]		
WAFER COAT[b]	**7G8[c]**	**MOSQUITO BBNP-40**	**MOSQUITO CHITINASE**
PBD Interference	34.96	6.87	0.0
TC 3X Ellipsometric	44.24	7.66	0.0

[a] Five microliter samples assayed 3 minutes at 45° C.
[b] Anti-*P. falciparum* MAb PF2A10 [100 ug/ml].
[c] Sample of the stock 7G8 sporozoites containing CS antigen.

The mosquito samples extracted in a buffer containing chitinase actually gave negative gray scale values. The enzyme may be degrading the ligand layer. A sample of strain 7G8 was incubated for four hours at RT in chitinase buffer. The sample yielded no visual signal when assayed 3 minutes at 45° C on the above TC 3X ellipsometric wafer. The chitinase treated samples may need to be treated to remove or inactivate any remaining active chitinase.

PERIOD 4:

Coating/Immobilization:

Polybutadiene polymer surfaces and wafers coated with Sera-Coat beads were generated as described in the lyme Ag detection period 3 section. Monoclonal antibody anti-*P. falciparum* [100 ug/ml in PBS, pH 7] were coated on the wafers as described in the period 2. Sera-Coat or PBD wafers coated with either MAb anti-*P. falciparum* were blocked for two hours at room temperature (RT) in PBS/1% BSA/2% sucrose/1% glycerol.

Sample Preparation:

The following buffers were evaluated in the extraction of uninfected mosquitoes:

A) blocking buffer(BB) - PBS/1% BSA/0.5 % Casein/0.01% thimerosal/0.002% phenol red

B) blocking buffer/Nonidet P-40 (BBNP-40) - BB/0.5% NP-40

C) BB/chitinase (0.28, 0.56, 1.12, 2.24 units/ml)

D) BB/0.5% NP-40/chitinase (0.28, 0.56, 1.12, 2.24 units/ml)

E) BB/NP-40 (0.062%, 0.125%, 0.25%, 0.5%)/chitinase (0.28 units/ml)

F) BB/NP-40 (0.062%, 0.125%, 0.25%, 0.5%)/chitinase (1.12 units/ml)

G) BB/0.1% NP-40/chitinase (0, 1.12, 2.24 units/ml)

H) BB/0.031% NP-40/chitinase (0.28 units/ml)

I) BB/0.062% NP-40

J) BB/0.031% NP-40

Mosquitoes (head/thorax), or whole ticks, were individually ground up in 0.05 ml of BB (with or without NP-40). The pestle was rinsed with additional buffer then the appropriate volume of chitinase (3.33 units/ml in 0.1 M sodium citrate, pH 5.5) was added. The samples were incubated overnight at room temperature with gentle agitation. The sample extracts were centrifuged for 5 minutes and the supernatant transferred to a new microcentrifuge tube.

Assay Conditions:

All the results reported were from samples assayed at 45° C for three minutes.

RESULTS:

DETECTION OF MALARIA ANTIGEN
EXTRACTED UNINFECTED MOSQUITO SAMPLES

EXTRACTION BUFFER	PBD* GRAY SCALE	SERA-COAT* GRAY SCALE
A - Blocking Buffer (BB)	44.39	13.91
C - BB/chitinase (0.28 units/ml)	33.60	13.88
C - BB/chitinase (0.56 units/ml)	12.78	10.92
C - BB/chitinase (1.12 units/ml)	19.02	7.08
C - BB/chitinase (2.24 units/ml)	19.60	3.79
P. falciparum strain 7G8 (in PBS) (1,280,000 sporozoites/ml)	35.50	19.95

*Wafers coated were interference.

An uninfected mosquito triturated in extraction buffer (B) when assayed on PBD and Sera-Coat wafers yielded negative gray scale values. The uninfected mosquito samples extracted in buffer (D) - BB/0.5% NP-40 with increasing concentrations of chitinase (0.28-1.68 units/ml) yielded negative gray scale values when assayed on both the PBD and Sera-Coat wafers. The samples prepared in buffer (G) likewise gave negative results on both wafers. Similarly, the two sets of uninfected mosquito samples extracted in buffers (E) and (F) yielded negative gray scale values when assayed on both the PBD and Sera-Coat wafers with the exception of those shown below.

DETECTION OF MALARIA ANTIGEN
EXTRACTED UNINFECTED MOSQUITO SAMPLES

EXTRACTION BUFFER	PBD* GRAY SCALE	SERA-COAT* GRAY SCALE
E - BB/0.062% NP-40/chitinase (0.28 units/ml)	0.99	Negative
H - BB/0.031% NP-40/chitinase (0.28 units/ml)	21.30	5.01
I - BB/0.062% NP-40	19.62	8.41
J - BB/0.031% NP-40	32.39	17.24

*Wafers coated were interference.

Negative gray scale values indicate that incubation of those samples lead to destruction of the surface probably due to removal of adsorbed antibodies in the case of PBD wafers, and removal of entire beads in the Sera-Coat wafers. The data clearly indicate that a balance must be struck between nonspecific binding and destruction of the surface. Chitinase or NP-40 alone both influence the level of background binding but cannot completely eliminate it without surface destruction. The optimal mixture at this time appears to be blocking buffer containing 0.062% NP-40 and 0.28 units/ml chitinase. Examination of this extraction media with infected mosquitoes must now be optimized to maintain maximal response.

PERIOD 5:

Coating And Immobilization:

Protocols were as described in previous periods for coating SERA coat wafers or PBD.

Assay Conditions:

Protocols were as described in previous periods.

Sample Preparation:

The following buffers were evaluated in the extraction of uninfected and infected mosquitoes:

A) Blocking Buffer (BB)

B) BB/chitinase (0.28 units/ml)

C) BB/0.062% NP-40

D) BB/0.062% NP-40/chitinase (0.28 units/ml)

E) BB/0.062% Tween 85

F) BB/0.062% Tween 85/chitinase (0.28 units/ml)

G) BB/0.062% Tween 20

H) BB/0.062% Tween 20/chitinase (0.28 units/ml)

Mosquitoes (head/thorax) were individually ground up in 0.05 ml of buffer. The pestles were rinsed with additional buffer. Buffers B,C, and D had 0.025 ml of chitinase (3.33 units/ml in 0.1 M sodium citrate, pH 5.5) added before being brought up to their 0.3 ml final volume with buffer. The samples were incubated overnight at room temperature with gentle agitation. The sample extracts were centrifuged for 5 minutes and the supernatant transferred to a new microcentrifuge tube.

Ab-Bead Conjugation:

A volume of MAb anti-*P. falciparum* sufficient to yield a final concentration of 20 ug/ml was activated and coupled to Sera-Coat beads as described above in lyme Ag detection period 5 section. Uninfected *An. freeborni* mosquitoes and *P. falciparum* (NF54) >90% infected *An.*

freeborni mosquitoes received from Dr. Wirtz were extracted as described above. Both uninfected and infected mosquitoes extracted in buffers A, B, C, F, G and H when assayed on PBD-MAb-anti-*P. falciparum* coated wafer gave positive results. The samples extracted in buffers containing Tween 20 and Tween 85 when assayed on Sera-Coat wafers for 3 minutes at RT yielded results similar to those seen with NP-40 in the buffer. The samples give negative gray scale values suggesting destruction of the surface due to removal of the beads from the Sera-Coat wafers.

DETECTION OF MALARIA ANTIGEN
UNINFECTED/INFECTED MOSQUITOES EXTRACTED IN BUFFER D
(PBD Gray Scale)

	#1	#2
Uninfected	Negative	Negative
Infected	21.75	12.74
P. falciparum strain 7G8 (in PBS)	31.06	-

PERIOD 6:

Monoclonal antibody anti-*P. falciparum*-conjugated Seradyne beads were prepared simultaneously with the MAb anti-*B. burgdorferi*-conjugated beads. Again one fraction was dialyzed against PBS, pH 7.0, to remove the carbodiimide. Dialyzed and undiallyzed beads were diluted 1:5 in PBS, heat-inactivated *P. falciparum* strain NF54 and CS recombinant protein $R32tet_{32}$. Five microliter samples assayed for 5 minutes at RT on a PBD interference wafer coated with MAb anti-*P. falciparum* were negative. An aliquot of the heat-inactivated *P. falciparum* strain NF-54, two uninfected mosquitoes and two infected mosquitoes (both extracted in BB/0.062% NP-40/chitinase (0.028 units/ml)) were incubated in a 56° C water bath for 3.5 hours. Five microliter samples assayed for 15 minutes at RT on a PBD interference wafer coated with MAb anti-*P. falciparum* produced no visual signals. A hyponitrous acid cellular extraction method was performed on a 20 ul aliquot of strain NF54 as described above in the lyme antigen detection period 6 section. The samples gave negative results when 5 ul were assayed for 15 minutes at RT on a PBD interference wafer coated with MAb anti-*P. falciparum*. Strain NF54 was diluted 1:2 in PBS/1% NP-40, PBS/1% Tween 20, and PBS/1% Tween 85. When these preparations, along with the PBS/surfactant diluents, were assayed for 15 minutes at RT on a PBD interference wafer coated with MAb anti-*P. falciparum* again only the two samples containing Tween 85 gave visual signals.

Samples of *An. freeborni* mosquitoes extracted in BB/0.062% NP-40/chitinase (0.028 units/ml) yielded negative results when assayed on PBD or Sera-Coat interference wafers coate with MAb anti-*P. falciparum* whether assayed 3 minutes at 45° C or RT for varying periods of time. It was hypothesized that proteolytic activity in the extracted samples was denaturing antigenic determinants.

Ten microliters of the following preparations were pipetted into wells cut into a 2% agarose/0.05% casein gel then placed into a 37° C, humidified incubator for 16 hours:

1 - BB/0.062% NP-40
2 - BB/0.062% NP-40/2% PMSF (phenylmethylsulfonyl fluoride - protease inhibitor)
3 - BB/0.062% NP-40/chitinase (0.028 units/ml)
4 - BB/0.062% NP-40/chitinase (0.028 units/ml)/2% PMSF
5 - Infected mosquito extracted in BB/0.062% NP-40/chitinase (0.028 units/ml)/2% PBSF

SUMMARY:

The following has been demonstrated for the malaria antigen detection system:
1. Heat inactivated organisms are easily detected.
2. Extraction protocols for the uninfected mosquitos can be developed and can be differentiated from the inactivated organisms. Addition of chitinase would appear to be an requirement.
3. PBD surfaces appear to offer the best performance of those examined to date.
4. Assay performance is best at 45°C.

Subsequent studies will include:
1. Optimization of the extraction protocol for improved resolution between infected and unifected mosquitos.
2. Increased ligand density.
3. Improve assay temperature performance.
4. Improve blocking protocols.
5. Improve the amplifying reagent.
6. Establish relevant sensitivity range.

MALARIA ANTIBODY DETECTION

The negative controls employed in this model were serum samples obtained from individuals employeed at BioStar. The positive control was a *P. falciparum* species specific IgG monoclonal antibody (MAb) Pf2A10[2].

PERIOD 2:

ANTIGEN COATING PROTOCOLS AND RESULTS:

For malaria Ab detection, R32tet 32 (20 ug/ml) was coated on to the PBD wafers using the fifth immobilization method described above. In testing these ellipsometric wafers, an in-house normal human sera sample was used as a negative control. The anti-*P. falciparum* MAb in water/glycerol did yield a visible signal when assayed on a wafer coated with R32tet32 in contrast to the observation made with the immobilized antibody and R32tet32 in water/glycerol. A fresh anti-*P. falciparum* sample reconstituted in DI water gave a 70% stronger signal versus the DI/glycerol sample when read on the comparison ellipsometer, data shown below.

MAb PF2A10 Reaction with R32tet32 Coated Wafer

Diluent	Gray Scale
DI/glycerol	31.48
DI water	53.48
PBS	92.97
Normal Human Serum[a]	7.55

[a] The human serum was used as a negative control, only.

The R32tet32 coated wafers were also examined with 5 ul of MAb anti-*B. burgdorferi* to examine specificity of the coated wafer. Given the successful results generated on the ellipsometric PBD wafers, the coating techniques and assay protocols were transferred to interference PBD wafers using the fifth immobilization technique described above. The blocking, sample, temperature and incubation conditions were all held constant.

SAMPLE	GRAY SCALE
Normal Human Serum	8.32
MAb anti-*B. burgdorferi* (H5332 in PBS)	5.20
MAb anti-*P. falciparum* (PF2A10 in PBS)	76.53

A preliminary examination of the reproducibility of the Mab interaction with the R32tet32 wafer surface gave the results shown below:

SAMPLE	AVE GRAY SCALE	S.D.	CV(%)
Normal Human Serum	8.01	0.41	5%
PF2A10	75.61	17.84	24%

This data suggests that the coating uniformity of the slides was poor using the fifth protocol described above, however, the blocking protocol is working very well for these surfaces. A titration of the PF2A10 antibody in PBS was also performed and the results given below:

SAMPLE	GRAY SCALE
Normal Human Serum	8.32
Direct PF2A10	76.53
PF2A10 (2x dilution)	26.83
PF2A10 (4x dilution)	25.13
PF2A10 (8x dilution)	14.08
PF2A10 (16x dilution)	9.33

PERIOD 3:

Coating/Immobilization: See Period 3 Lyme Antigen Detection above.

Assay Conditions:

As mentioned above, preliminary investigations of other systems had optimized the assay conditions for five microliter samples incubated for three minutes on a 45° C heating block. However, the malaria Ab detection assay gave favorable results when assayed for 5 minutes at room temperature (RT). Additionally, assays run at 15 minutes in a humidified, 37° C incubator were performed for the assay but were less than optimal.

For malaria Ab detection, $R32tet_{32}$ (83 ug/ml) was coated on to the wafers using the methods described above. In testing these ellipsometric wafers, two in-house normal human sera samples (60426 and JL) were used as negative controls. The anti-P. *falciparum* MAb was the positive control. Initially, wafers with a polybutadiene (PBD) reactive surface were compared with Sera-Coat bead wafers.

DETECTION OF MALARIA ANTIBODY

WAFER COAT	ASSAY PROTOCOL*	MAb GRAY SCALE	60426 GRAY SCALE	JL GRAY SCALE
PBD (Interference)	45° C, 3 minutes	79.86	10.00	47.61
Sera-Coat TC 3X (Ellipsometric)	45° C, 3 minutes	62.71	5.15	34.63
Sera-Coat TC 3X (Ellipsometric)	RT, 5 minutes	43.94	0.00	0.00

* Sample applied was a 5 microliter sample.

BioStar would prefer to design a triprobe assay which could be run at room temperature. There would then be no need for any additional equipment or power sources. The results in the preceding table suggest this goal maybe attainable. Further experiments examining assay conditions were conducted and are discussed below. The effect of incubating the Ab/Ag Sera-Coat wafers for 30 minutes in a 37° C, saturated glutaraldehyde (GA), vapor incubator was examined. Glutaraldehyde (GA) covalently cross-links proteins by their amino groups. Finally, the two different Sera-Coat beads, TC 3X and TC 7X, were compared under parallel conditions.

DETECTION OF MALARIA ANTIBODY - SERA-COAT WAFERS
GA-TREATED VS NON-GA-TREATED
TC 3X VERSUS TC 7X

WAFER COAT*	ASSAY PROTOCOL	MAb GRAY SCALE	60426 GRAY SCALE	JL GRAY SCALE
TC 7X	45° C, 3 minutes	88.03	2.28	15.98
TC 7X (GA)	45° C, 3 minutes	83.60	4.73	13.13
TC 3X	45° C, 3 minutes	62.71	5.15	34.63
TC 3X (GA)	45° C, 3 minutes	81.62	9.65	28.07
TC 7X	RT, 5 minutes	19.60	9.07	15.54
TC 7X (GA)	RT, 5 minutes	31.26	11.57	9.8
TC 3X	RT, 5 minutes	43.94	0.00	0.00
TC 3X (GA)	RT, 5 minutes	21.63	0.0	7.03

* Wafers coated were ellipsometric.

The gray scale values generated by all three samples are larger when assayed at 45° C. There were no clear distinctions between the GA-treated and non-GA-treated assay values. It is doubtful that there are any beneficial reasons for treating these wafers with GA. The sera negative control samples yielded lower values on the TC 3X versus the TC 7X wafers when assayed at RT for 5 minutes.

In a further effort to reduce the gray scale values of the negative control sera samples in the malaria antibody detection assay wafers were incubated for two hours at RT in the following blocking solutions:

1) PBS/1% human serum albumin
2) PBS/5% normal human sera
3) PBS/5% chicken sera/0.1% Tween 20/0.1 mM thimerosal

Activated Sera-Coat TC 3X ellipsometric wafers were coated with $R32tet_{32}$ [83 ug/ml]. Five microliter samples of anti-*P. falciparum* MAb, and the negative control serum samples were assayed at 45° C for 3 minutes.

DETECTION OF MALARIA ANTIBODY
BLOCKING REAGENTS

BLOCKING AGENT	MAb GRAY SCALE	60426 GRAY SCALE	JL GRAY SCALE
#1	56.96	3.16	16.85
#2	68.64	9.85	1.38
#3	80.90	26.87	13.47

There was no improvement in the gray scale values for the negative control serum samples. Therefore, we continued to use PBS/1% BSA/2% sucrose/1% glycerol as the blocking reagent.

PERIOD 4:

Coating/Immobilization:

Polybutadiene polymer surfaces and wafers coated with Sera-Coat beads were generated as described above. $R32tet_{32}$ [80 ug/ml in PBS] were coated on the wafers as described above. Sera-Coat or PBD wafers coated with $R32tet_{32}$ were blocked for two hours at room temperature (RT).

Assay Conditions:

The malaria Ab detection assay has been performed under various conditions, applying samples both directly with a pipette and using an applicator pad. Normal human serum (NHS) samples were assayed on PBD wafers coated with the CS recombinant protein $R32tet_{32}$, however, the sample bleeds when rinsed after incubation. This creates an abnormally high background value which interferes with the exact reading of the sample spot. Increased incubation times at lower temperatures, sample applicators, addition of the detergent Tween 20 to the sample and blotting samples before rinsing were examined. Blotting the sample was the most successful method in alleviating this problem, however, this adds an additional undesirable step in the assay. Sera-Coat wafers did not display this bleeding effect and retained reactivity.

DETECTION OF MALARIA ANTIBODY

WAFER	NHS GRAY SCALE	MAb GRAY SCALE
Sera-Coat	0.00	45.62

5 ul assayed for 5 minutes at RT

A time course study was performed incubating 5 ul of MAb anti-*P. falciparum* on a PBD interference wafer coated with $R32tet_{32}$ [80 ug/ml] at RT for varying periods of time.

DETECTION OF MALARIA ANTIBODY
PBD GRAY SCALE

	15 (m)	10 (m)	5 (m)	1 (m)	30 (s)	15 (s)
MAb	53.56	62.13	54.74	33.92	16.20	18.86

Four-fold serial dilutions of the MAb anti-*P. falciparum* (0.5 mg total protein/ml PBS, pH 7.0) were prepared in PBS/1% BSA/2% sucrose/1% glycerol (1:4, 1:16, 1:64) were assayed, on a PBD interference wafer coated with $R32tet_{32}$, for 5 minutes at RT. Results of intraassay and interassay variability, representing data collected from four assays performed three different PBD wafers utilizing two separate serial dilutions, are presented in the following table.

MALARIA ANTIBODY DETECTION

ASSAY #		ANTIBODY CONCENTRATION 8 µg/ml	31 µg/ml	125 µg/ml	500 µg/ml
	MEAN	6.1	29.8	53.0	60.7
1	SD	1.8	2.2	2.0	0.9
	%CV	30.0	7.3	3.8	1.5
	MEAN	6.0	27.7	52.5	55.7
2	SD	1.1	2.8	0.4	6.6
	%CV	17.9	10.1	0.8	11.8
	MEAN	7.5	32.6*	55.8	61.2
3	SD	0.8	0	1.6	3.2
	%CV	10.4	0	2.9	5.2
	MEAN	6.2	24.4	57.1	60.1
4	SD	1.3	2.7	1.8	2.1
	%CV	21.6	11.3	3.2	3.5
	MEAN	6.4	28.2	54.2	59.2
INTERASSAY	SD	1.3	3.5	2.7	3.9
	%CV	19.8	12.5	4.9	6.6

* Value based on one sample.

SUMMARY:

We have demonstrated in the Phase I study the following:
1. Clear differentiation of the monoclonal antibody to the malaria antigen from the antibody to lyme antigen or normal human serum.
2. A titer of 1:64 for the monoclonal antibody provided. It should be noted that an actual human serum with antibodies to malaria antigens has not been examined and would be expected to perform better than the monoclonal antibody as the affinity will be higher.
3. Again PBD is the preferred surface, this is the desired endpoint for the production of a panel assay. All analytes that are to be examined must be compatiable with a common surface for immobilization of the respective ligands.
4. Room temperature assays are preferrable to the elevated temperatures as the normal human sera give a lower level of binding under these conditions.
5. Assay times of greater than 5 minutes do not substantially increase the detection of the antibody.
6. Good reproducibility as a function of concentration is obtained, for the current level of development. With the inter-assay performance being much better than would be anticipoated for this stage of development.

Future Studies:
1. Increase ligand density.
2. Clinicals.

3. Alternate Surface Preparations.
4. Sample Diluents.

PHASE I CONCLUSIONS:

We have demonstrated that Optical ImmunoAssay provides a viable format for the simultaneous detection of multiple disease states and/or multiple infectious agents. As observed in previous assay systems, detection of host Ab responses is more readily accomplished than direct antigen detection. The primary limiting factor in both cases is the surface density of the reactive sites. This is a less severe constraint on antigen immobilization than it is for antibody immobilization as the orientation upon immobilization is less critical. In general, antigens possess more reactive sites per molecule than do antibodies. This fact corresponds to our observation that antigen detection systems tend to saturate earlier than corresponding antibody detection systems. We are confident that these problems may be overcome by more thorough investigation into the molecular nature of surface immobilization for these systems.

Optical ImmunoAssay clearly presents significant improvements over current immunological testing methods. Protocols for assay performance are exceptionally simple, usually requiring only samle application. This allows a non-expert to easily interpret both a visual and instrumented result. These characteristics are ideally suited to both field and laboratory use.

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